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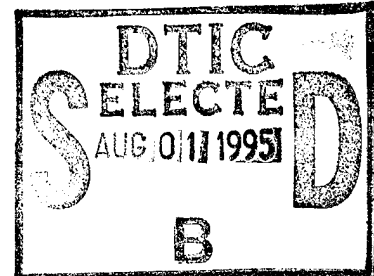
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Ronald S. Calverton
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Introduction

Statement of Work for Original Grant. Construct the high-level expression vectors for the anthrax toxin genes. Construct, using recombinant DNA procedures, an asporogenic *B. anthracis* which shall be used for these recombinant DNA experiments. Mutate sporulation-specific genes so that when these genes are inserted back into these bacteria, these organisms will then be unable to form spores. For toxin gene expression, transfer the T7 RNA polymerase gene (from plasmid pAR1173) into the *E. coli-Bacillus* shuttle plasmid pSI-1, to allow for the regulated expression in the *Bacillus* species of the cloned toxin genes. A large DNA fragment, containing the T7 RNA polymerase gene linked to its IPTG-inducible promoter shall be inserted into an *E. coli* plasmid lacking a functional *Bacillus* replicon. Convert the recombinant plasmid into an integration plasmid, using *B. anthracis*-specific DNA, and then insert into the asporogenic *B. anthracis* for the stable expression of the T7 RNA polymerase gene.

Construct *Bacillus* plasmids containing each of the anthrax toxin genes, and transform these plasmids into *B. anthracis* for toxin gene expression. Following treatment with IPTG, T7 RNA polymerase shall be expressed; transcribe the toxin genes located downstream from the powerful T7 promoter in these plasmids. Monitor the production of toxin and characterize the recombinant toxin proteins using standard toxin assays. Although the T7 RNA polymerase-based expression system is anticipated to produce large quantities of intact protein in *B. anthracis*, also construct recombinant plasmids for the constitutive (non-inducible) expression of the individual toxin proteins, especially PA for use in animal vaccine studies. Conduct and complete experiments designed to determine nucleotide differences in the toxin genes from the virulent *B. anthracis* compared to the avirulent Sterne strain using PCR and SSCP electrophoresis techniques. These procedures allow for the

detection of single nucleotide differences in DNAs which are 300-400 base pairs in size.

Preliminary Research and Background. Prior to the initiation of the current research program, the cloning, sequencing and initial characterizations of the EF (*cya*) and LF (*lef*) genes were performed at BYU (1,3,4,6). The PA gene (*pag*) was cloned and sequenced in the Bacteriology Division at USAMRIID (2,5,9). Schematic diagrams of these three toxin genes are shown in Figure 1, along with partial restriction maps. PA, apparently due to the presence of its active promoter, has been expressed in high levels in *B. subtilis* as well as in *B. anthracis* (9,22). In contrast, neither EF nor LF has been expressed at such high levels, probably due to weaker promoters, although the PA promoter has been linked to these genes for higher levels of expression, but the level attained was not equivalent to that of PA (unpublished data of D. Robertson). Even though large quantities of PA can be obtained from culture supernatants of cultures containing *B. anthracis*, it is desirable that each of the individual toxin proteins be prepared in the absence of the other proteins, to avoid the production of active toxin, for safety benefits, and for being able to study the biochemistry of these proteins individually.

Construction of an integration plasmid which expresses T7 RNA polymerase. The first approach that we employed for the production of a plasmid that expresses T7 RNA polymerase was to insert the appropriate DNA fragments into pSI-1 (19,21,23; see Figure 2), which replicates in both *Bacillus* and *E. coli* strains. This plasmid grows to chloramphenicol resistance due to the presence of the CAT (chloramphenicol acetyl transferase) gene from pC194. However, in order to integrate the T7 gene into the *B. anthracis* genome, the functional *Bacillus* replicon would have to have been removed 20). We initially felt that experiments could be performed using this self-replicating plasmid, but our first results using this plasmid, along with a plasmid containing the toxin genes, proved to be

difficult in *B. anthracis*. One of these problems was due to plasmid instability, probably since both plasmids contained the same *Bacillus* replicon (from pUB110; see Figure 4), although each plasmid had different antibiotic resistance genes (CAT or kanamycin resistance). In addition, we also encountered difficulties in isolating plasmid from *E. coli* (especially strains deficient in *dam* methylation, such as SCS110) which could then be transfected directly into the different *B. anthracis* strains. As a consequence, we changed our host vector for the expression of the T7 RNA polymerase into one which we produced in our lab. This plasmid (designated P126; see Figure 7) which contains the CAT gene from pC194 (as a *HpaII* to *Sau3A* DNA fragment) inserted into *DraI* cleaved pUC12. The pUC12 was cleaved with *DraI* which removes most of the coding region for the ampicillin resistance gene leaving a DNA with blunt ends. After ligation and transformation into competent *E. coli*, the transformation mix was grown on a chloramphenicol-containing (10 µg/ml) agar plate in the presence of IPTG and X-gal to allow for the selection of *E. coli* which retain a functional α -peptide gene (for blue-white color selection of recombinants) and a functional CAT gene. This plasmid cannot grow in the *Bacillus* species since it lacks a functional *Bacillus* replicon. It can be grown in *E. coli* to chloramphenicol resistance, although growth is slower, apparently due to slow induction of CAT activity in *E. coli*.

The T7 RNA polymerase gene was inserted into the multicloning region of pSI-1 using the following strategy. First, the *EcoRI* to *BamHI* DNA fragment from pSI-1 (Figure 2 and 3), which contains the *spac*-1 phage promoter upstream of a functional *lac* operator to allow for regulated expression in the presence of IPTG, was cloned into a modified pUC8 DNA. This *EcoRI* to *BamHI* DNA, derived from pSI-1, also contains the *lacI* repressor gene which is transcribed constitutively from the penicillinase promoter. The pUC8 DNA was modified by removing its *PstI* cleavage site

by cutting pUC8 with *Pst*I and producing blunt-ends with T4 DNA polymerase. The resulting plasmid (designated pUC8Pst) is not cleaved by *Pst*I. This is important because there is a *Pst*I site in the multicloning sequence (MCS) of pSI-1. The *Pst*I site in the pSI-1 MCS will be used for cloning the T7 RNA polymerase gene after the MCS is inserted into pUC8Pst. The pUC8Pst DNA that we produced was cleaved with *Eco*RI and *Bam*HI prior to ligation to the *Eco*RI to *Bam*HI fragment from pSI-1. After ligation, the DNA mixture was transformed into competent *E. coli* and individual colonies isolated. The resulting plasmid (pFM1; see Figure 5) was then used to link the T7 RNA polymerase gene up to the *spac*-1 promoter.

The T7 RNA polymerase gene, from pAR1173 (17,18; see Figure 6) was cloned into pBluescript SK+ using *Bam*HI, which removes the entire T7 RNA polymerase gene as a single DNA fragment. The orientation of the T7 RNA polymerase gene was such that the *Pst*I recognition site, within the recombinant plasmid, was downstream of the 3'-end of the T7 RNA polymerase coding region. Immediately upstream of the start codon, a *Xba*I restriction enzyme cleavage site was inserted using site-specific mutagenesis procedures. After mutagenesis, the T7 RNA polymerase gene was removed as a *Xba*I to *Pst*I DNA fragment and inserted into pFM1, which was cleaved with the same enzymes. The resultant plasmid, pFM2 (see Figure 5), was used as the source of DNA for the T7 RNA polymerase gene used in the integration vector.

Initially, we planned to modify pFM2 directly by introducing the kanamycin resistance gene from pUB110 into this plasmid, to allow for antibiotic selection. In addition, we would also need to insert *B. anthracis* genomic DNA into this plasmid for integration and stable expression of the T7 RNA polymerase gene. However, we were not successful in producing a DNA fragment from pUB110 using PCR (11,12) which retained kanamycin resistance. The reason for this problem is not

known, but apparently involved the selection of PCR primers. This failure also delayed for several months the production of the integration vector. Therefore, we decided to use the CAT gene in the integration vector, since we were also having problems using the CAT gene in the toxin gene shuttle vectors. The kanamycin resistance gene could then be used for the toxin gene expression vectors using a new DNA construct which we made in our laboratory.

pFM2 was cleaved with *EcoRI* and *SaI* to produce a DNA fragment containing the regulated promoter driving the synthesis of the T7 RNA polymerase gene as well as containing the *lacI* gene. P126 (see Figure 7) was cleaved with the same enzymes and the two DNAs ligated together. The resulting plasmid, designated P126-TL (see Figure 7), contains a functional CAT gene for antibiotic selection, the T7 RNA polymerase gene under the control of the *spac-1* promoter and the *lac* operator, and the *lacI* gene, which is transcribed from the penicillinase promoter. This plasmid was then ready to be modified for integration into the *B. anthracis* genome. In order to facilitate this integration event, *B. anthracis* DNA was first cleaved with *EcoRV* to produce blunt-ended DNA. *EcoRV* was chosen since its recognition sequence (GATATC) has a similar GC% to *B. anthracis* genomic DNA. The *EcoRV* cleaved *B. anthracis* DNA was ligated into P126-TL at its unique *SmaI* cleavage site. Following ligation, the total DNA mixture was transformed into competent *E. coli* (*dam*⁻; SCS110 from Stratagene) and grown on chloramphenicol-containing agar plates. DNA was then isolated from these bacteria and used for electro-transformation of *B. anthracis*. After electroporation, the transformed *B. anthracis* were plated onto chloramphenicol-containing agar plates and individual colonies selected. These bacteria were used for toxin gene expression.

Construction of the toxin gene expression and shuttle plasmid. The plasmid that we eventually ended up using for the toxin gene shuttle plasmid is derived from pUBUC18 (see Figure

8) which serves as an efficient *Bacillus-E. coli* shuttle vector. This plasmid contains both *Bacillus* and *E. coli* replicons and was constructed in our laboratory using pUB110 and pUC18. First, pUB110 was cleaved with *EcoRI* and *BamHI*. After this digestion, the cleaved DNAs were treated with T4 DNA polymerase to fill in the 5'-sticky ends to produce blunt-ended DNA molecules. This DNA mixture was then ligated into *DraI*-cleaved pUC18. (As stated above, *DraI* cleaves within the coding region of the ampicillin resistance gene leaving a DNA containing blunt ends.) After ligation and transformation into competent *E. coli*, the bacteria were plated onto a kanamycin-containing (30 µg/ml) agar plate in the presence of IPTG and X-gal to allow for the selection of *E. coli* containing a functional α -peptide gene (for blue-white color selection of recombinants) as well as the functional kanamycin resistance gene. This plasmid, since it contains all of the replication regions from pUB110 (*rep* protein, *oriU*, and *palU*), will grow in the *Bacillus* species. Several blue colonies were selected, all having identical properties. Figure 8 shows the restriction map of pUBUC18.

The multicloning region from pET21a, which contains the T7 RNA polymerase promoter, the T7 RNA polymerase terminator, a functional *E. coli* ribosome binding site (rbs), as well as several unique restriction enzyme cleavage sites, was cloned into the pUBUC18 MCS. First, however, PCR primers which flank the *BglII* recognition site and a mutagenic oligonucleotide, which introduces an *EcoRV* cleavage site (see Figure 9), were used to produce a DNA product using PCR. This DNA was cloned into pCRScript-Direct (Stratagene) at the unique *SrfI* (or *SmaI*) site. Once an *E. coli* clone containing the pET21a MCS was identified (verified by PCR using the original primers), this DNA region was removed using *BglII* and *EcoRV* and cloned into pUBUC18, with some modification. First, pUBUC18 was cleaved with *NdeI*, which cleaves downstream from the MCS (at nucleotide number 1380) and immediately upstream from the *palU* replication domain (at nucleotide

number 2261), leaving both regions intact. After digestion, the DNA was treated with T4 DNA polymerase to produce blunt ends. Then, this DNA was treated with *Bam*HI. The *Bgl*II to *Eco*RV fragment containing the pET21aMCS was then inserted into the modified pUBUC18 DNA, allowing for the *Bgl*II and *Bam*HI ends to ligate, as well as the *Eco*RV and blunted-end *Nde*I site to combine. The resulting plasmid, which has some of the non-essential DNA from pUBUC18 removed (between the two *Nde*I sites), is designated pDR181 (see Figure 10). This plasmid serves as the toxin gene shuttle vector. The toxin genes were inserted downstream of the T7 RNA polymerase promoter in the multicloning region.

Using the assumption that the endogenous ribosome binding sites (rbs) immediately upstream of the toxin gene opening reading frames function well in *B. anthracis*, we decided to use the intact rbs for each gene instead of using the same rbs, or a synthetic rbs, for each toxin gene. In fact, data reported by other investigators (29) suggest that the more nucleotides in the rbs which are complementary to the 3'-end of the 16S rRNA, the better these genes are translated in the *Bacillus* spp. Under this assumption, and assuming that *B. anthracis* has the same sequence as *B. subtilis* at the 3'-end of its 16S rRNA, then the sequence would be 3'-UUUCCUCCACAUG-5'. The presumed rbs for LF and for PA is 5'-AAAGGAG-3', whereas for EF, the rbs is 5'-AAAGGAGGU-3'. The longer stretch of complementary nucleotides present in the EF rbs suggests that its rbs will be the most efficient in initiating translation, at least in the *Bacillus* spp. In addition, there appears to be an inverse correlation that exists for efficient *E. coli* rbs, where longer stretches of complementary nucleotides may actually inhibit translation initiation in *E. coli*. Since the rbs present in the MCS region of the pET21a insert which is present in pDR181 has been optimized for *E. coli* translation, we felt that a better *Bacillus* rbs should be used in our anthrax toxin gene vectors. Therefore, we

decided to use the endogenous toxin gene rbs for each gene.

Using PCR, each of the *B. anthracis* toxin genes was mutated to introduce a unique *Xba*I recognition site immediately upstream from the endogenous rbs of the respective gene. Cleavage at this restriction site will allow for positioning of the appropriate gene downstream from the T7 RNA polymerase promoter, and will replace the *E. coli* rbs in the MCS with the appropriate *B. anthracis* rbs in the toxin gene expression vectors. It is important to note, that the *B. anthracis cya* gene has a *Xba*I recognition site in its coding region (see Figure 1), but since the sequence of this particular *Xba*I site is part of a 5'-GATC-3' sequence which is methylated on the adenine residue by the *dam* methylase, cleavage will not occur at this interior site when the DNA is grown in *dam*⁺ strains of *E. coli*. The downstream PCR primer for each toxin gene contained another appropriate restriction enzyme site for directional cloning. However, the *lef* gene has a second *Xba*I recognition site outside of the coding region for LF, but within the region which is amplified by PCR. Consequently, directional cloning using two different enzymes was not possible using the *lef* gene fragment, but the direction of the resulting clone in toxin gene shuttle vector was verified for this gene as well as the other two genes, even though only one direction of the inserted DNA is possible. Moreover, the nucleotide sequence for each of the toxin genes was determined after PCR cloning to ensure that no mutations were introduced during amplification.

Once the appropriate DNA fragment for each of the toxin genes was identified, these DNAs were cut with *Xba*I and either *Bam*HI for the PA gene or *Nde*I for the EF gene. Of course, as mentioned above, the LF gene was removed as a single *Xba*I DNA fragment. Once these DNA fragments were produced, they were cloned into pDR181, which was also cut with the same pairs of enzymes. After ligation, the DNA mixtures were transformed into competent *E. coli* using

electroporation for most transformations although chemically competent cells could also be used. After sequencing and restriction mapping confirmed that the appropriate toxin gene was inserted into the shuttle vector, these vector DNAs were used to transform *B. anthracis*. Plasmids pDR182, pDR183, and pDR184 contained the LF, PA and EF genes, respectively. Diagrams showing the restriction maps of these plasmids are shown in Figures 11, 12, and 13.

Preparation of *B. anthracis* Genomic DNA. In order to produce integration plasmids described above for the stable insertion of the inducible T7 RNA polymerase gene in *B. anthracis*, it was necessary to prepare *B. anthracis* genomic DNA. The procedure that we have used to isolate genomic DNA has been under development in our lab for many years (8,10). The current protocol which yields that best DNA so far is described here. *B. anthracis* strain UM44 (obtained from the laboratory of Dr. Curtis Thorne) was grown overnight in L-Broth to saturation density. The cells were pelleted by spinning at $10,000 \times g$ in a Sorvall HB-4 rotor for 10 minutes. The cell pellet was resuspended in TNE buffer (25 mM Tris-HCl [pH 8.0]; 100 mM NaCl; 10 mM EDTA) containing 1 mg/ml lysozyme and incubated for 60 minutes at 37°C. The cell suspension was clearly lysed judged by an increase in viscosity. SDS was added to give a final concentration of 2% and proteinase K was added to a final concentration of 100 µg/ml. The solution was then incubated overnight at 37°C. The DNA solution was extracted at least three times with equilibrated phenol-chloroform (1:0.5). The DNA, present in the aqueous phase, was eventually spooled from a 70% ethanol solution containing 0.3 M NaOAc [pH 5.2]. The DNA fibers were washed in 95% ethanol, dried using a KimWipe and dissolved in TE buffer (10 mM Tris-HCl [pH 7.5]; 1 mM EDTA). Several milligrams of pure genomic DNA were prepared from a single 100 ml culture.

Construction of an Asporogenic *B. anthracis*. When this research program was begun,

there were no asporogenic *B. anthracis* available. Therefore, we undertook experiments designed to use recombinant DNA techniques to produce *B. anthracis* strains which fail to form spores. Our initial proposal involved taking advantage of the precise, synchronized onset of sporulation (24,25) in order to isolate RNA which might be specific for sporulation (26,27). However, the approach that we ended up using was based on gene homologies which might exist between sporulation-dependent genes of *B. subtilis* and *B. anthracis*. For example, the *spo0H* gene, which encodes a sigma-30 transcription factor, is absolutely required for sporulation in *B. subtilis*. Since there is a large difference in GC% between *B. anthracis* DNA (around 30% GC) and *B. subtilis* (approximately 40-45% GC), we did not feel that oligonucleotides for specific regions of a *B. subtilis* gene would be specific enough for hybridization. Therefore, we used PCR to produce a DNA fragment that could be used for hybridization. The *spo0H* gene from *B. subtilis* (30) was used to identify a related gene in *B. anthracis*. Using PCR, we produced a DNA molecule from the *spo0H* gene for use in hybridization experiments. The oligonucleotide primers used were G339: 5'-GTG AAT CTA CAG AAC AAC AAG GG-3' and G340: 5'-GCG AGT AGC TGT CTT TAT TGC GG-3'. The position of these primers is shown in Figure 14. The selection of primers, and their position in the coding region, was based on homology comparisons between the *spo0H* protein product and other transcription factors. We selected a region of the gene which was not homologous to other known transcription factors, so that our hybridizations would not just identify *B. anthracis* transcription factors but a gene truly homologous to the *B. subtilis spo0H* gene which would likely perform the same function in the onset of sporulation in *B. anthracis*.

Using the PCR-produced DNA, we made complementary DNA which was radioactive and hybridized this DNA to Southern blots containing both *B. subtilis* as well as *B. anthracis* DNA

cleaved with several different enzymes. Based on initial hybridization data, we observed that this radioactive *spo0H*-specific DNA detected unique DNA bands in digests prepared from both *B. subtilis* and *B. anthracis* DNA. For *B. anthracis*, a single DNA band was always present in the lane digested with *EcoRI* which was approximately 4.5 kbp in size. A similar band, but slightly larger was also present in the lanes digested with *HindIII*. Therefore, we used *EcoRI* and *HindIII* to cleave *B. anthracis* DNA and combined this cleaved DNA with bacteriophage λ ZAP-Express which had also been cleaved with *EcoRI* or *HindIII*. After ligation, some of the ligated DNA was packaged using the λ packing lysate from Stratagene. The packaged bacteriophage was plated on agarose plates containing *E. coli* XL1. After an overnight growth, phage DNA was transferred onto nylon membranes and hybridized to the *spo0H*-specific DNA, which had been previously used for the Southern blot. After the development of the X-ray film, several positive plaques were identified. After several rounds of hybridization, six lambda isolates continued to hybridize to the *spo0H*-specific DNA. At the present time, these clones need to be sequenced to determine if an open reading frame is present which corresponds to the open reading frame for the *B. subtilis spo0H* gene. If a positive comparison can be determined, then the corresponding *spo0H* gene in *B. anthracis* cells can be mutated.

Analysis of the Toxin Genes from Different *B. anthracis* Isolates. In an effort to determine whether the individual toxin genes from different *B. anthracis* strains have differences compared to the Sterne strain, we sequenced the EF and LF genes from several different virulent strains. The rationale behind these experiments is based on the fact that certain strains of mice (15,16,28) are killed by Sterne *B. anthracis* whereas most mice and other animals can be safely injected with these bacteria. It is possible that the Sterne strain, which has been extensively

propagated in the lab may contain sequence variations compared to the wild-type, virulent strains. We were initially going to use single-stranded (ss) conformational polymorphism (SSCP) electrophoresis (13,14) to detect any nucleotide differences in the toxin genes. However, we found out that we could just as easily perform a DNA sequence analysis directly using our automated fluorescent DNA sequencer of the individual toxin genes from different *B. anthracis* strains. Using the oligonucleotide primers that were used for initial sequence determination of these genes, we were able to determine the entire gene sequence for each toxin gene in a single run of the 373A ABI automated DNA sequencer.

In order to analyze DNA from different *B. anthracis* strains as rapidly as possible, we developed a preparation protocol for the isolation of whole cell DNA from different *B. anthracis* strains. This protocol, which is a slight modification of the procedure that we also developed for the preparation of large quantities of pXO1 and pXO2, yields enough DNA for PCR and other small-scale analyses. *B. anthracis* is grown overnight in L-Broth or other rich media. After stationary growth is attained, 1.5 ml of the cultured bacteria is removed and transferred to a 1.5-ml microcentrifuge tube. The tube is centrifuged at top speed in a microcentrifuge for 1 min. The supernatant is discarded and the pellet is resuspended in 0.20 ml of E-buffer (0.04 M Tris-acetate [pH 7.9], 2 mM EDTA) by vortexing until fully resuspended. To the suspended bacteria, 0.40 ml of *freshly* prepared lysis buffer (40 ml prepared using 0.3 g Tris-base; 7.5 g sucrose; 0.18 M NaOH; 3% SDS) is added and the solution is vortexed thoroughly. The mixture is incubated in a water bath maintained at 65°C (the solution should be cloudy and somewhat viscous) for 30 min, vortexing every 5 to 10 min. After this incubation, the tube is centrifuged for 10 min at top speed in a microcentrifuge at 4°C. The clear, yellow supernatant is carefully removed to a new tube and

neutralized by adding 0.10 ml of cold 2.0 M Tris-HCl [pH 7.0]. Then, 0.5 ml of equilibrated phenol-chloroform (1:0.5) is added and the phases mixed by vortexing. After a centrifugation to separate the phases, the upper aqueous phase is removed. An equal volume of isopropanol is added, mixed, and incubated on ice for 60 minutes. The tube is then centrifuged to pellet the nucleic acid. After removing the alcohol, the nucleic acid pellet is dissolved in 200 µl TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). The DNA can be stored indefinitely at 4° C.

Using the DNA isolated using the small-scale protocol described above, we initially performed PCR to produce copies of the entire toxin gene. Oligonucleotides, which are located outside the protein coding region for each toxin gene, were used for PCR. These primers are shown below:

Primers for PCR of the entire anthrax toxin gene:

LF: G342 5'-GAA ATG TTC ACT CTA GAA AAA AGG-3'
G345 5'-ATT AAA TCC ATA TGA AAA TTT TTA ATA CAT-3'

PA: G350 5'-ATA AAT TTA ATT TTC TAG AAA AAG GAG AAC G-3'
G358 5'-GGA TCC TAC AAA CAA TCT CAA AGG-3'

EF: G352 5'-GAA TAT CTA AAT ATC TAG AAC CAA AGG AGG-3'
G357 5'-CCC AAA GCA TAT GAA ACT GGG-3'

In order to assure that large quantities of full-length toxin gene were produced, we used the Taq Extender reagent from Stratagene that allows for long PCR products to be produced. In addition, to avoid the possibility of gene mutations caused by PCR, we sequenced the DNA directly after a PCR reaction so that we would have a mixture of sequences, most of which would have the wild-type nucleotide sequence, instead of producing a clone of the product and sequencing that clone. Moreover, the sequence of the toxin genes was determined from several PCR reactions as well, to

reduce any chance of incorrect nucleotide sequences.

We determined the nucleotide sequence of the EF and LF genes for the Vollum 1b, UM23, V770, Ames, NH, and Sterne strains of *B. anthracis*. We were not able to detect any differences in the sequences of these genes from any of these strains. This result was a little surprising, since nucleotide differences in different strains of pathogenic organisms do occur. Nevertheless, it appears that major nucleotide differences in the toxin genes are not responsible for the differences in virulence that is seen experimentally in the more pathogenic *B. anthracis* strains. It would still be interesting, however, to be able to analyze some of the strains isolated from disease outbreaks in isolated areas, especially since each of the strains used are also propagated in the laboratory, and have various degrees of virulence. While these experiments were being conducted in our laboratory, I found out from Dr. Welkos (USAMRIID) that they had also determined that no difference exists in the PA gene from different strains as well.

Conclusions

During the course of this research project we were able to construct vectors for the high-level expression of the anthrax toxin genes. Using the IPTG-inducible region from plasmid pSI-1, we inserted this region of DNA into a plasmid, designated P126-TL, which contains the T7 RNA polymerase gene inserted downstream from the *spac*-1 promoter, which is derived from the SPO bacteriophage. IPTG induces expression of the T7 RNA polymerase since the *lacI* repressor is inactivated and the bacterial RNA polymerase initiates transcription. This plasmid contains the CAT gene from pC194 which allows for antibiotic selection. In addition, a unique *SmaI* recognition site, located between the T7 RNA polymerase gene and the *lacI* gene has been used for insertion of a *B. anthracis* genomic DNA restriction fragment for integration into the genome. This plasmid lacks a functional *Bacillus* replicon.

The shuttle vector for expression of the individual anthrax toxin genes (pDR181) consists of the replication components of pUB110 as well as its kanamycin resistance gene. The MCS from pET21a, which contains the T7 RNA polymerase promoter and terminator, has been inserted into pDR181. Each of the individual anthrax toxin genes has been inserted into this plasmid at the unique *XbaI* recognition site downstream from the T7 promoter. After these recombinant plasmids are grown in *dam*- *E. coli*, these DNAs can be transferred directly into *B. anthracis* strains.

Using the expression vectors just described, we have produced *B. anthracis* which produce anthrax toxin protein. Unfortunately, the amount of protein has not yet been quantified, but LF was easily detected in the culture supernatant on a denaturing polyacrylamide gel. Toxin protein was also detected in the cellular pellet as well.

We have isolated at least six recombinant λ ZAP clones which contain *B. anthracis* DNA

sequences homologous to the *spo0H* gene of *B. subtilis*. The *spo0H* gene in the *Bacillus* species is absolutely required for sporulation to occur. Based on hybridization analysis, we used DNA derived from a part of the *spo0H* DNA sequence contained in the coding region for this protein, but which should not have homology to other known transcription factors. As a consequence, the region of DNA that we characterized should only be present in this gene and cross-hybridization with *B. anthracis* DNA indicates that a similar gene, having a similar DNA sequence has been isolated. We still need to sequence these DNAs to determine whether the analogous *spo0H* gene has been isolated in *B. anthracis*.

The individual anthrax toxin genes from several different virulent *B. anthracis* strains have been sequenced. Using a rapid DNA purification procedure, we performed PCR on these DNAs to isolate full-length toxin gene DNA fragments. After these DNAs were purified, DNA sequence analysis of them were performed using the automated 373A ABI fluorescent DNA sequencer. We observed that neither the EF or LF genes from several different virulent strains (Vollum 1b, UM23, V770, Ames, NH) had nucleotide differences compared to the Sterne strain of *B. anthracis*. Similar results have been reported by researchers at USAMRIID who sequenced the PA gene from several different *B. anthracis* strains as well. This results suggests that sequence variations in the toxin proteins cannot account for the difference in virulence of the different *B. anthracis* strains.

Refereed Publications During Research Period.

Up to this time, no manuscripts have been submitted for publication pertaining to the content of this research program. However, since the research pertaining to the T7 RNA polymerase-based expression system has been completed, a manuscript describing the results of this research will be written within the next couple months. Copies of this manuscript will be sent to USAMRDC as soon as they are completed.

Graduate Degrees Resulting from Grant Support.

At the present time, only two individuals, Marshall Mendenhall and Frank Spangler, will have included research data pertaining to this research grant as part of their graduate theses. Marshall Mendenhall completed his M.S. degree in 1994 and Frank Spangler will complete his M.S. degree in 1995.

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Figures

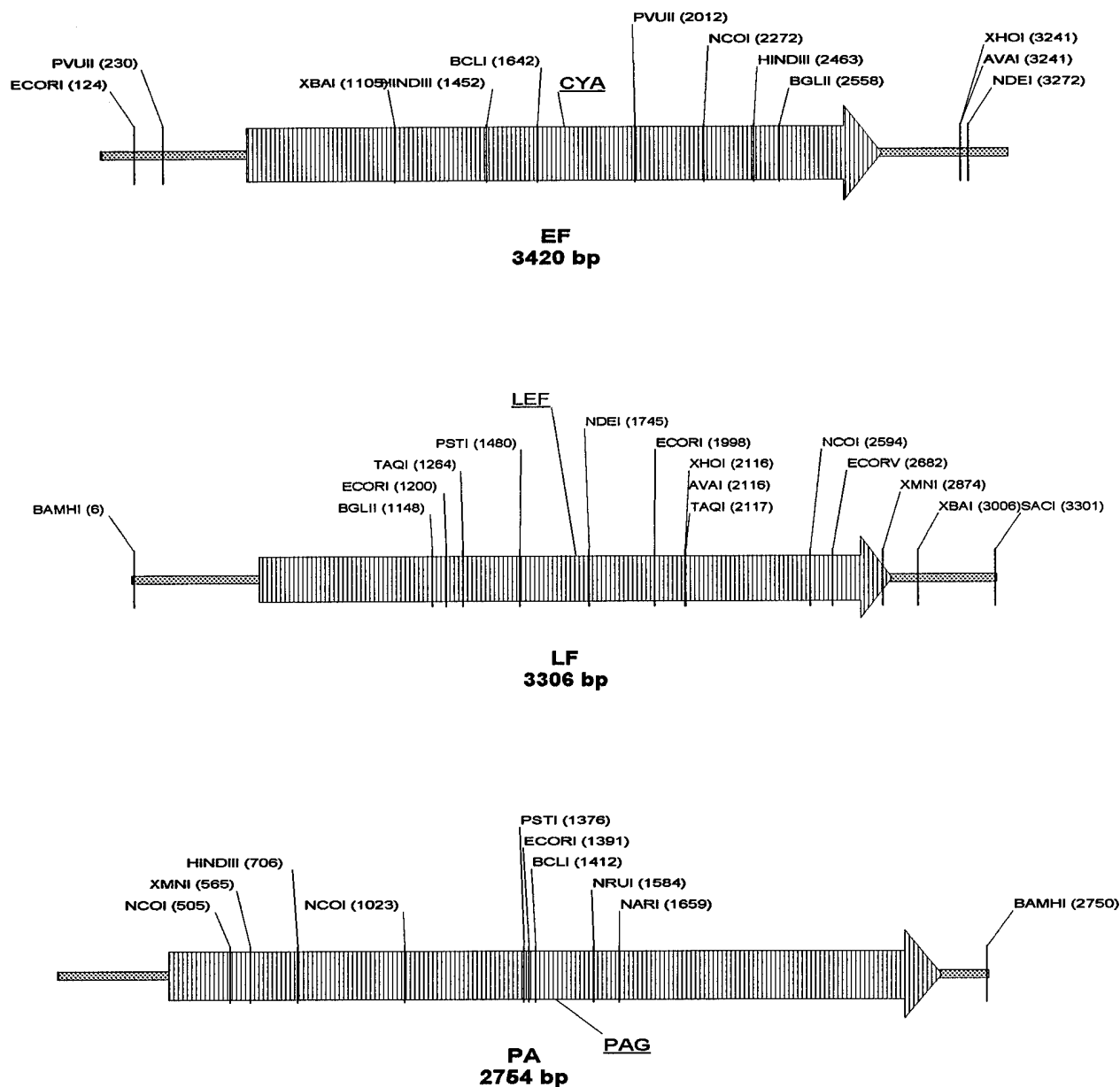


Figure 1. Partial restriction maps of the three anthrax toxin genes are derived from published nucleotide sequences (1,2,3,4,5,6,7).

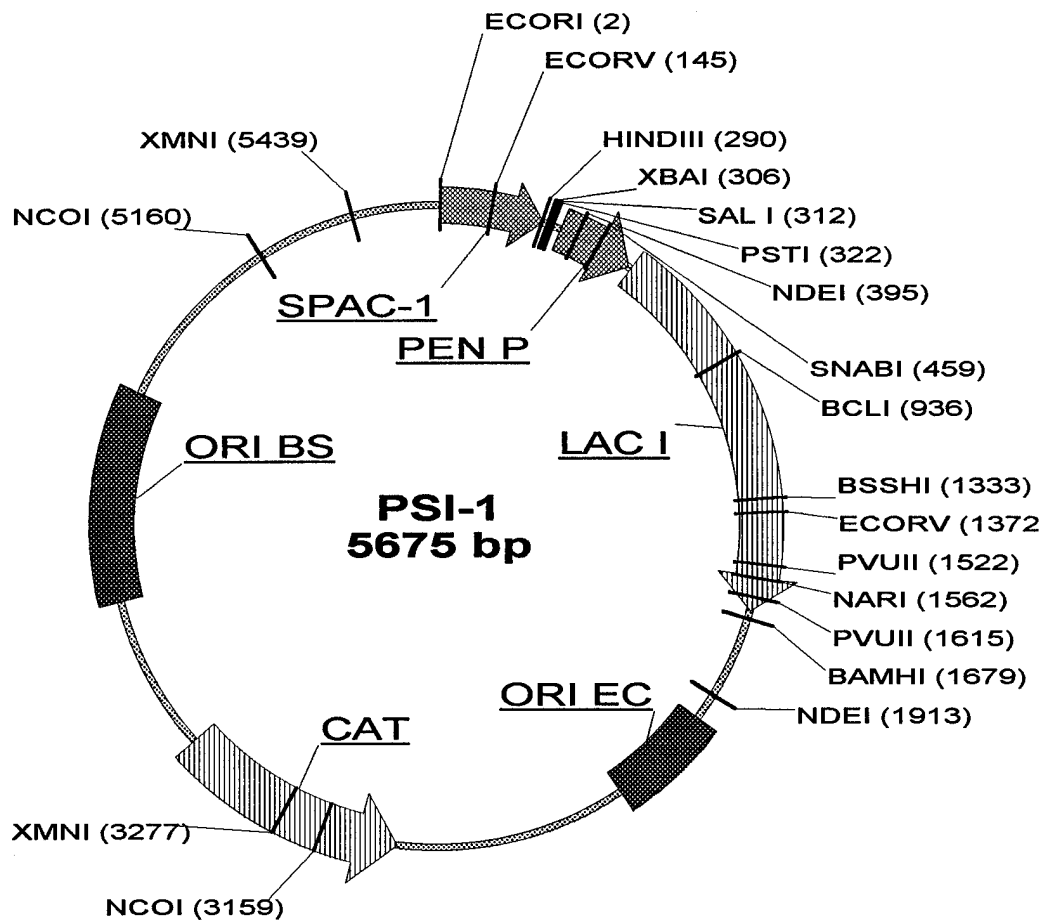


Figure 2. pSI-1 is the *B. subtilis* plasmid which allows for regulated gene expression from the *spac*-1 promoter in the presence of IPTG. This bifunctional shuttle vector allows for plasmid propagation in both *Bacillus* and *E. coli* strains. The CAT gene from pC194 can be used in both Gram- and Gram+ bacteria. The penicillinase promoter results in constitutive expression of the *lacI* repressor gene. The sequence of the multicloning region for this plasmid is shown in Figure 3.

Figure 3. Nucleotide sequence of the *spac-1* promoter. Also shown is the *lac* operator, transcription start site and cloning sites for plasmid pSI-1 (see Figure 1).

```

      EcoRI
1    GAATTCTACA CAGCCCAGTC CAGACTATTC GGCAGTGAAG TTATGGGTGA AGTGGTCAAG
61   ACCTCACTAG GCACCTTAAA AATAGCGCAC CCTGAAGAAG ATTTATTTGA GGTAGCCCTT

      EcoRV
121  GCCTACCTAG CTTCCAAGAA AGATATCCTA ACAGCACAAG AGCGGAAAGA TGTTTTGTTC
181  TACATCCAGA ACAACCTCTG CTAAAATTCC TGAAAAATTT TGCAAAAAGT TGTTGACTTT
                                     * -35 *

                                     +1 start site for RNA transcription
241  ATCTACAAGG TGTGGCATAA TGTGTGGAAT TGTGAGCGGA TAACAATTAA GCTTAAGGAG
                                     * -10 *      <<  lac operator  >>      * rbs *

      XbaI      PstI
301  GTGATCTAGA GTCGACCTGC AGGCATGCAA GCTAATTC

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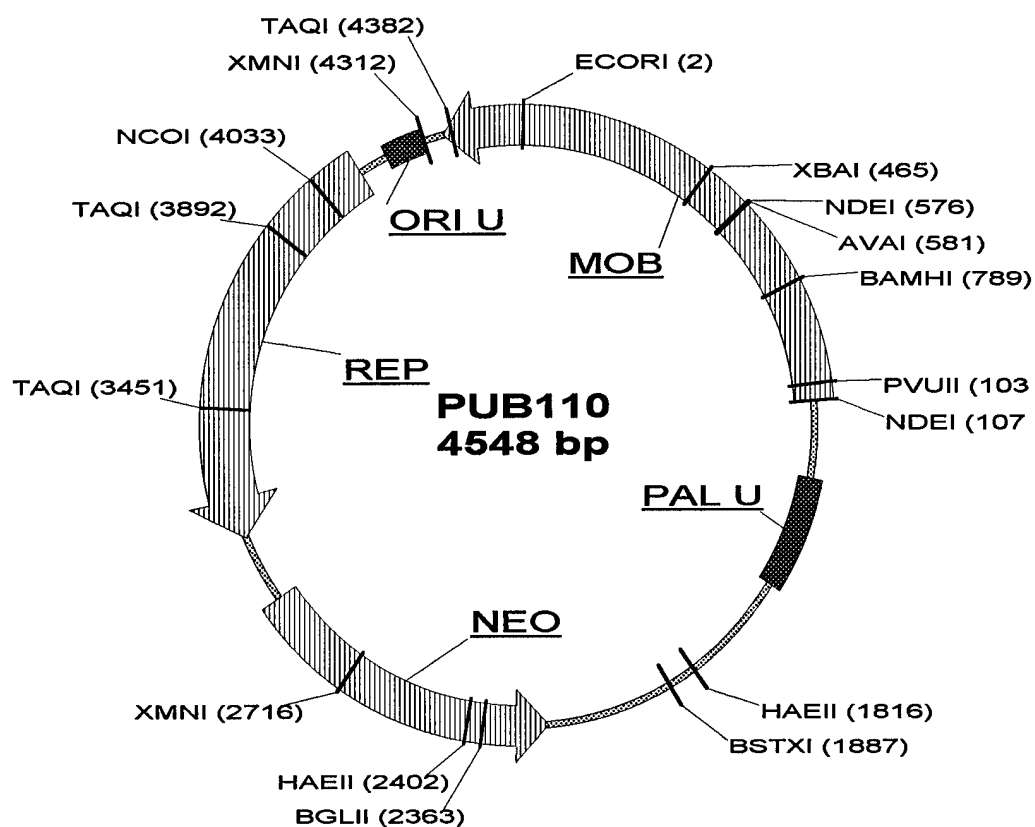


Figure 4. *Bacillus* plasmid pUB110. This plasmid contains the neomycin and kanamycin resistance gene for the shuttle vectors. The replication origin from pUB110 is in pSI-1, pUBUC18, pBS42 and other vectors.

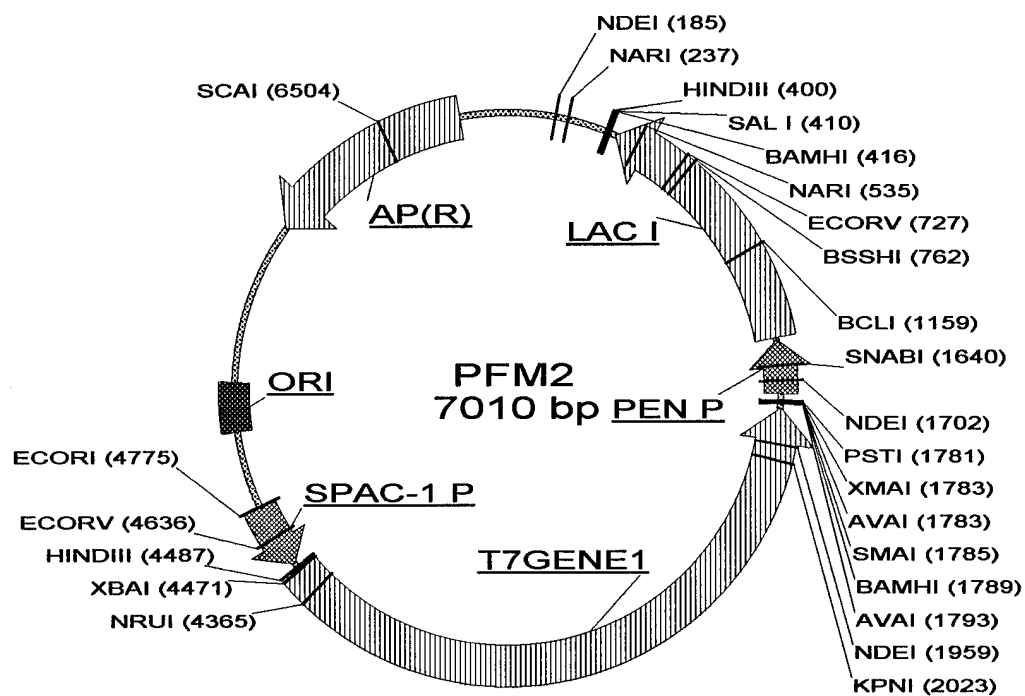


Figure 5. The T7 RNA polymerase gene inserted into the regulated *spac-1* promoter from pSI-1. The *lacI* gene is also produced. This plasmid is the source of these genes for the integration vector.

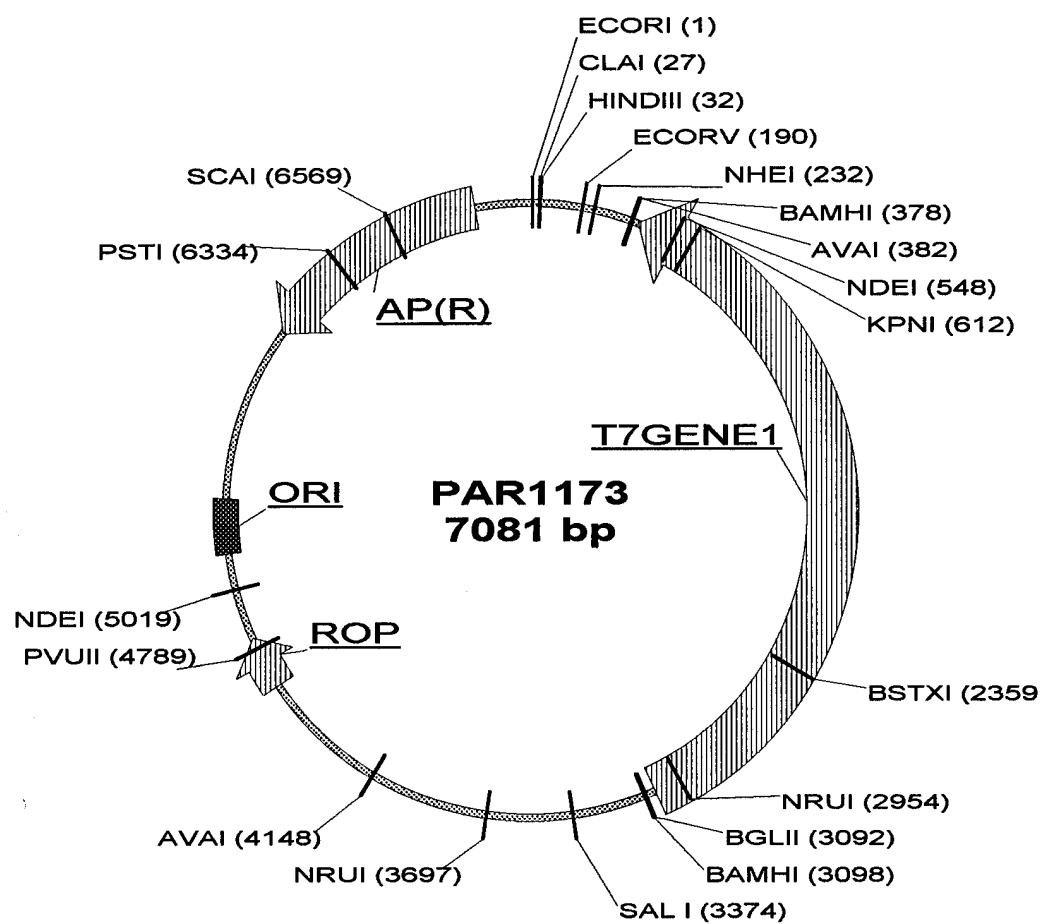


Figure 6. pAR1173 containing the T7 RNA polymerase gene.

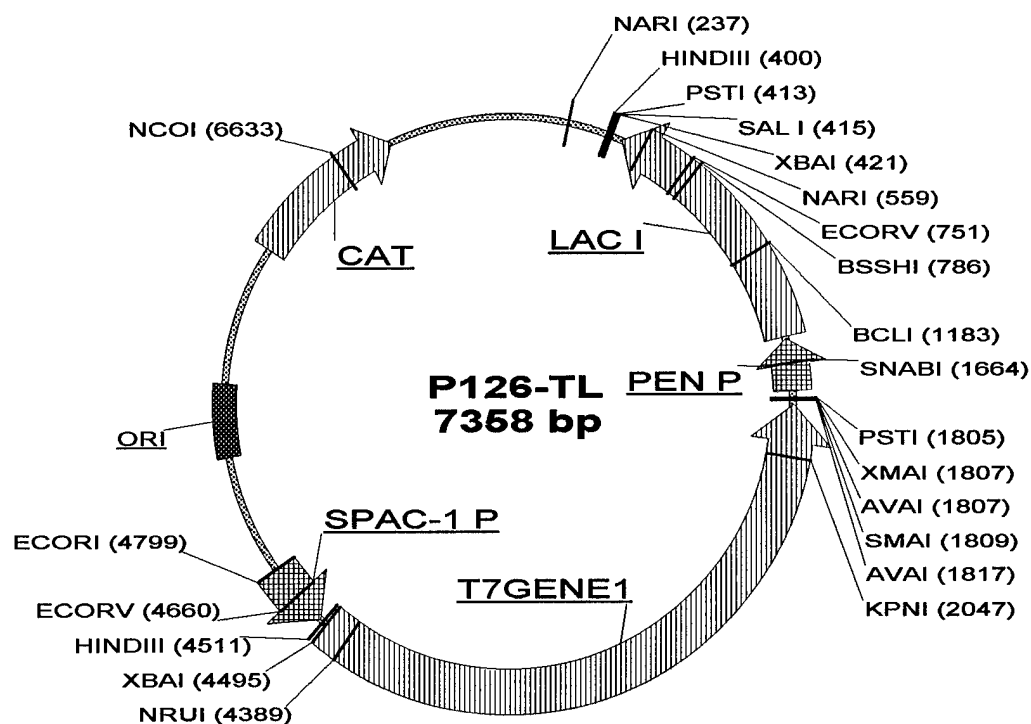


Figure 7. Plasmid P126-TL is derived from pUC12 and has the CAT gene from pC194 inserted into the *DraI* cleavage sites in the ampicillin resistance gene. The T7 RNA polymerase gene is inserted downstream from the *spac-1* promoter from pSI-1. In addition, *lacI* repressor is produced from the penicillinase promoter.

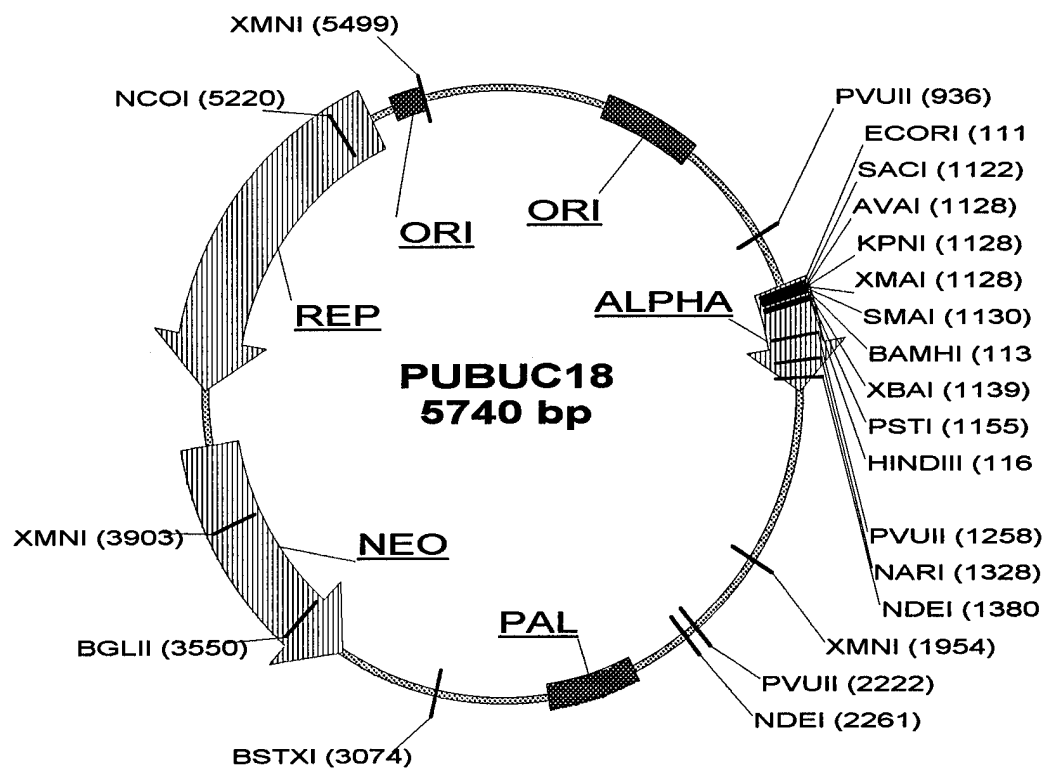


Figure 8. A shuttle vector for the *Bacillus* species and for *E. coli*. The *neo* gene can be used for both neomycin and kanamycin resistance. The intact α -peptide of *lacZ* allows for blue-white color selection in *E. coli*.

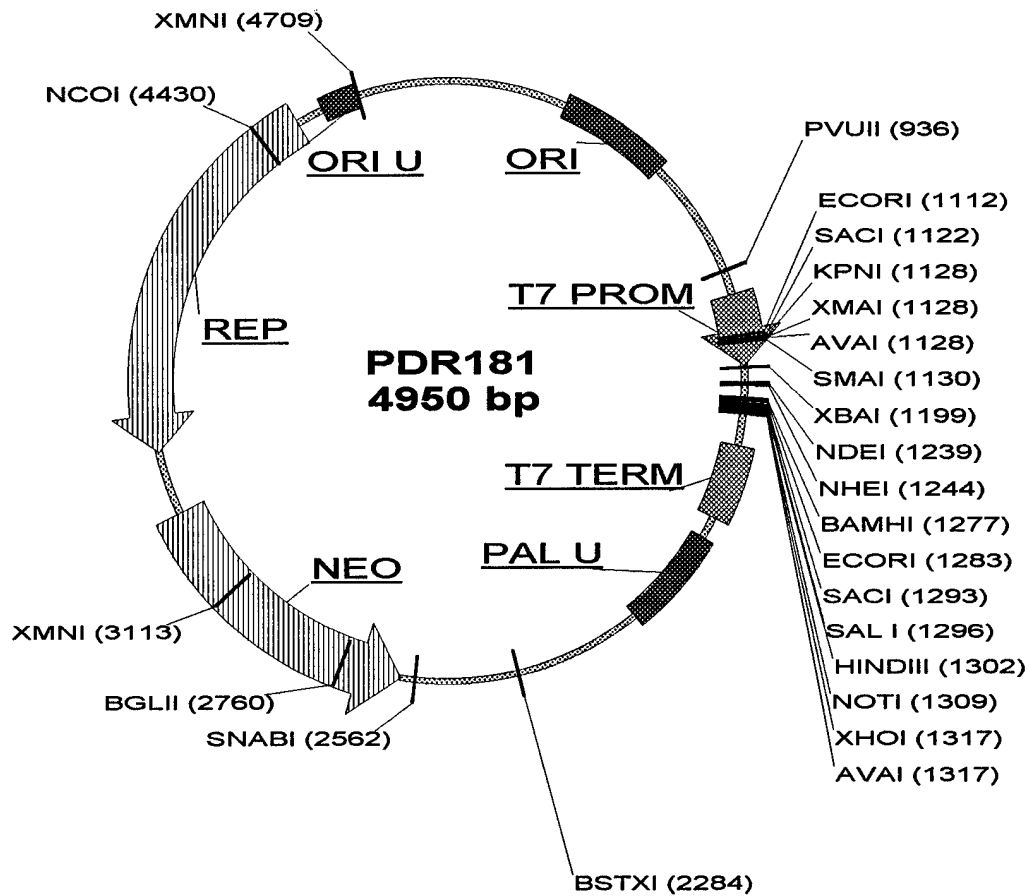


Figure 10. Anthrax toxin gene shuttle vector pDR181. The anthrax toxin genes can be inserted downstream of the T7 RNA polymerase promoter. Other genes can also be placed downstream from this promoter for expression in the *Bacillus* species as well.

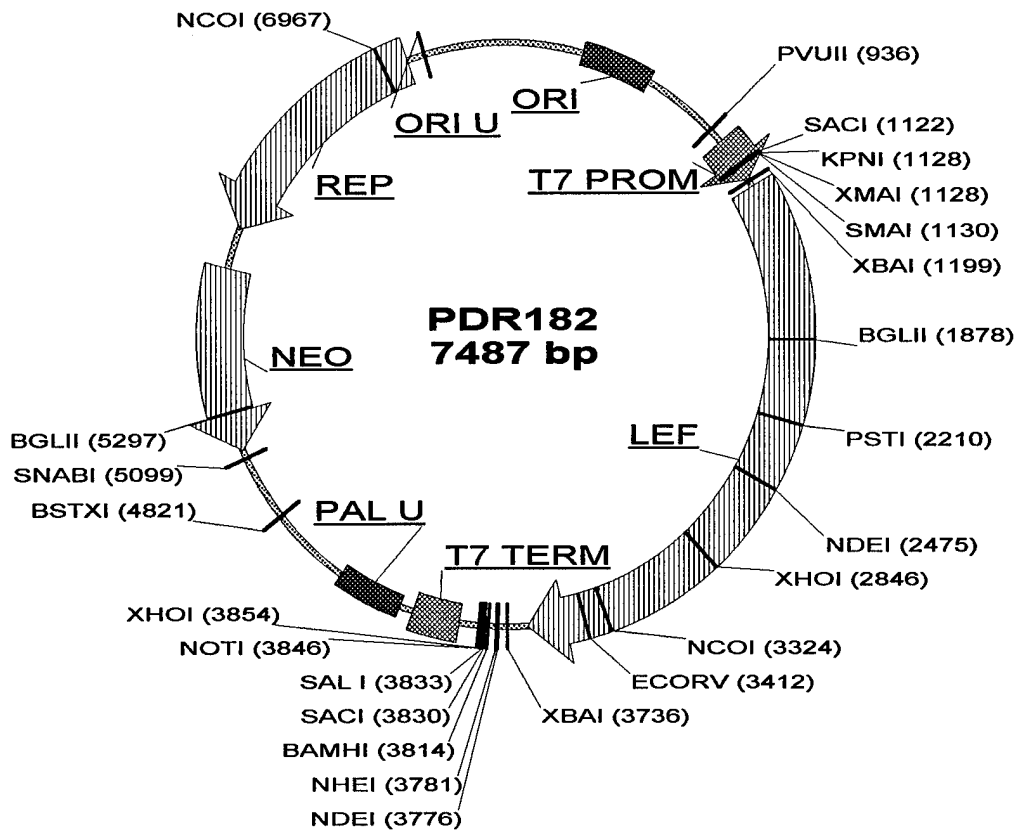


Figure 11. Anthrax toxin gene shuttle vector containing the *B. anthracis* lethal factor (*lef*) toxin gene.

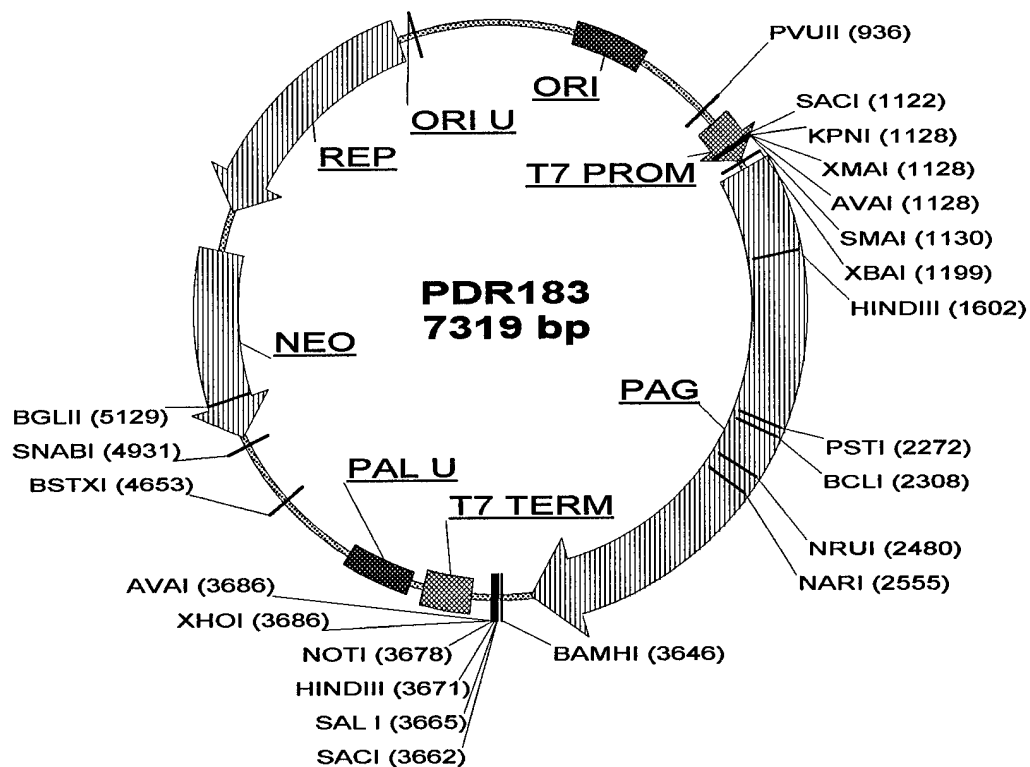


Figure 12. Anthrax toxin gene shuttle vector containing the *B. anthracis* protective antigen (*pag*) toxin gene.

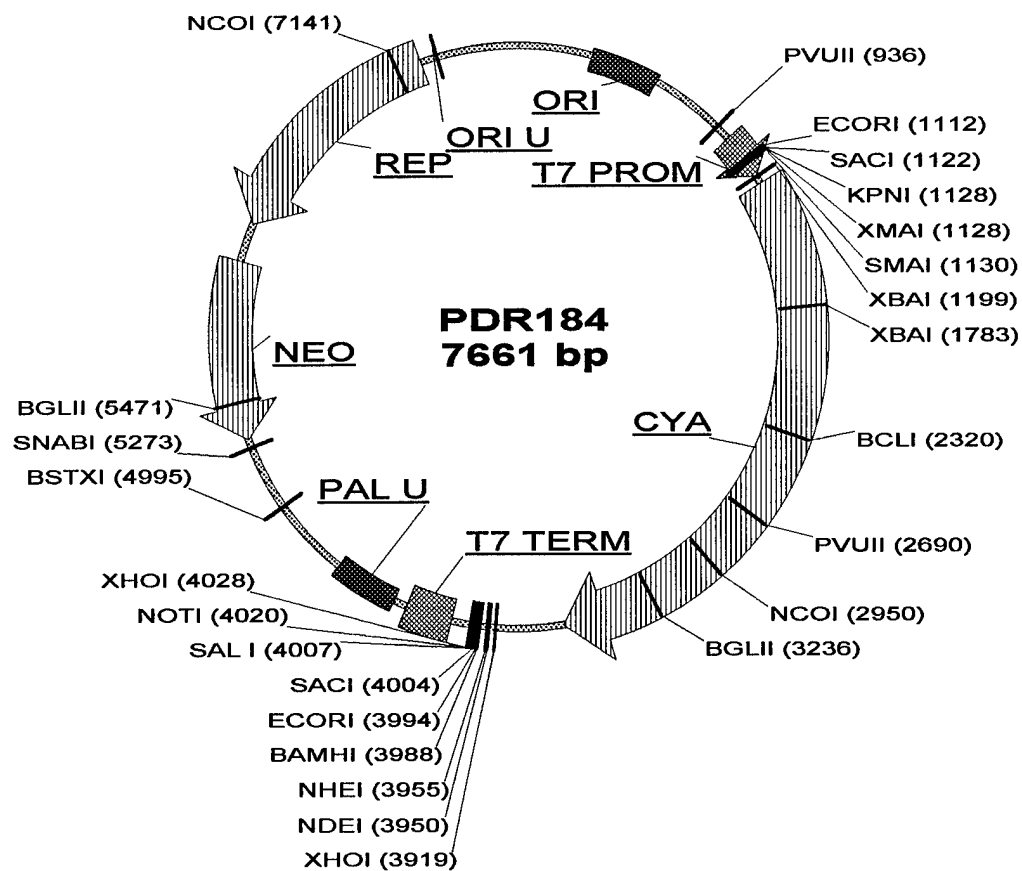


Figure 13. Anthrax toxin gene shuttle vector containing the *B. anthracis* edema factor (*cya*) toxin gene.

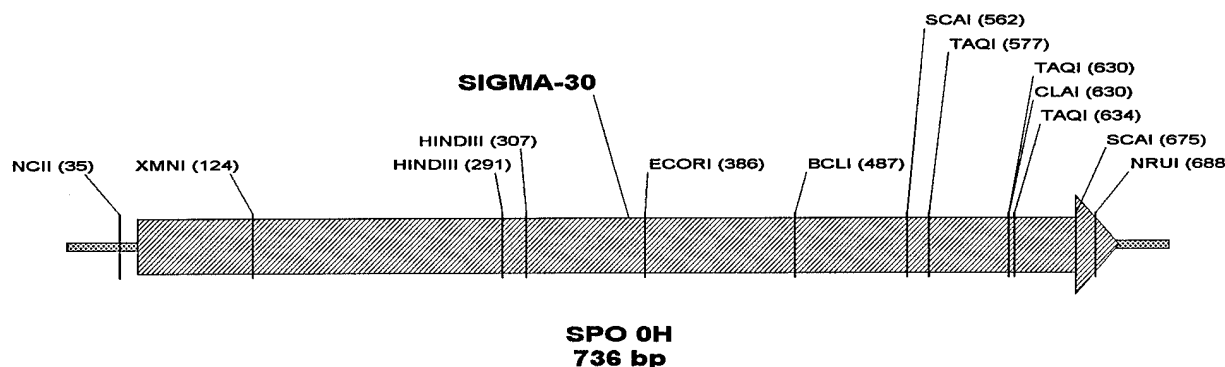


Figure 14. The nucleotide sequence for the *spo0H* gene, which encodes a sporulation specific sigma-30 factor required for transcription and entrance into sporulation. The nucleotides in bold and underlined correspond to the PCR primers (see text) used to produce DNA that was used as a probe to identify the homologous *B. anthracis spo0H* gene. The protein open reading frame is from nucleotide 47 through 703.

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1 ccaatactgt ataatatctt tatctacgtg cgccgggggg atcggaggtga atctacagaa
61 caacaagggga aaattcaaca aagagcagtt ttgccagttg gaggacgagc aggtcattga
121 aaagggttcat gttggggaca gtgatgcgtt agattacttg attacgaagt accgaaactt
181 tgttcgggca aaagcaagat cctatttctt aataggggag gacagagagg atattgttca
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301 attcaaagct tttgcagaat tatgtattac ccgccaaatt attaccgcgaa taaagacagc
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481 ggaaatgatc attaatacagg aagaatttga tgatattgaa atgaaaatgg gagaactatt
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721 ttgacagtat ttttct

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